Modulation and Plasticity of Rhythm-Generating Networks

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Chapter 3

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Prostaglandin E_2 differentially modulates the central control of eupnoea, sighs and gasping in mice

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Key points

- Prostaglandin E_2 (PGE_2) augments distinct inspiratory motor patterns, generated within the preBötzinger complex (preBötC), in a dose-dependent way. The frequency of sighs and gasping are stimulated at low concentrations, while the frequency of eupnoea increases only at high concentrations.
- We used *in vivo* microinjections into the preBötC and *in vitro* isolated brainstem slice preparations to investigate the dose-dependent effects of PGE_2 on the preBötC activity.
- Synaptic measurements in whole cell voltage clamp recordings of inspiratory neurons revealed no changes in inhibitory or excitatory synaptic transmission in response to PGE_2 exposure.
- In current clamp recordings obtained from inspiratory neurons of the preBötC, we found an increase in the frequency and amplitude of bursting activity in neurons with intrinsic bursting properties after exposure to PGE_2.
- Riluzole, a blocker of the persistent sodium current, abolished the effect of PGE_2 on sigh activity, while flufenamic acid, a blocker of the calcium-activated non-selective cation conductance, abolished the effect on eupnoeic activity caused by PGE_2.

Abstract Prostaglandins are important regulators of autonomic functions in the mammalian organism. Here we demonstrate *in vivo* that prostaglandin E_2 (PGE_2) can differentially increase the frequency of eupnoea (normal breathing) and sighs (augmented breaths) when injected into the preBötzinger complex (preBötC), a medullary area that is critical for breathing. Low concentrations of PGE_2 (100–300 nM) increased the sigh frequency, while higher concentrations (1–2 μM) were required to increase the eupnoeic frequency. The concentration-dependent effects were similarly observed in the isolated preBötC. This *in vitro* preparation also revealed that riluzole, a blocker of the persistent sodium current (I_{Nap}), abolished the modulatory effect on sighs, while flufenamic acid, an antagonist for the calcium-activated non-selective cation conductance (I_{CAN}) abolished the effect of PGE_2 on fictive eupnoea at higher concentrations. At the cellular level PGE_2 significantly increased the amplitude and frequency of intrinsic bursting in inspiratory neurons. By contrast PGE_2 affected neither excitatory nor inhibitory synaptic transmission. We conclude that PGE_2 differentially modulates sigh, gasping and eupnoeic activity by differentially increasing I_{Nap} and I_{CAN} currents in preBötC neurons.

Detailed description of author contribution:
HK conceived the idea to study Prostaglandin E2
HK, AG3 and JMR designed the experiments
HK performed the majority of the experiments CC, FE, SZ and AG3 added somemeasurements in brainstem slices
AD performed in vivo recordings in anesthetized animals
HK, CC and FE analyzed the data
HK and JMR wrote the paper

Abbreviations aCSF, artificial cerebrospinal fluid; AP, action potential; COX-2, cyclooxygenase-2; EMG, electromyography recordings; FFA, flufenamic acid; I_{CAN}, calcium-activated non-selective cation conductance; I_{Nap}, persistent sodium current; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; preBötC, preBötzinger complex; sEPSCs, spontaneous excitatory post synaptic currents; sIPSCs, spontaneous inhibitory postsynaptic currents

Introduction

Breathing has to adapt continuously and differentially to changes in the external and internal environment. At the centre of this adaptation lie neuronal networks that provide the flexibility to respond to ongoing changes. The preBötzinger complex (preBötC; Smith et al. 1991; Schwarzacher *et al.* 2011), a region that is essential for the generation of breathing (Ramirez *et al.* 1998; Wenninger *et al.* 2004; Tan *et al.* 2008), has been implicated in the modulatory response of breathing. This region, located within the ventrolateral medulla, is the target of several neuromodulatory systems (Doi & Ramirez, 2008, 2010; Koch *et al.* 2011; Ramirez *et al.* 2012). In addition to the classic neuromodulators such as noradrenaline (norepinephrine) (Ellenberger *et al.* 1990; Viemari & Ramirez, 2006; Zanella *et al.* 2014), substance P (Gray *et al.* 1999; Peña & Ramirez, 2004); acetylcholine (Shao *et al.* 2005) and serotonin (Peña & Ramirez, 2002; Ptak *et al.* 2009), this network is also regulated by molecules that can be activated through non-neuronal pathways. Some of these molecules are part of the inflammatory pathway and include prostaglandins, which are produced by cyclooxygenase-2 (COX-2) enzymes. Under physiological conditions, these inducible enzymes are expressed at basal levels in the brain. But a number of stimuli, including peripheral infections, pain, traumatic injury, hypoxia and hyperoxia, raise COX-2 expression and subsequently increase protein levels of prostaglandin E_2 (Yamagata *et al.* 1993; Perez-Polo *et al.* 2011). PGE_2, the major reaction product of the COX-2 enzymes, has been implicated in directly modulating the neuronal activity involved in several regulatory systems, including the regulation of pain (Ahmadi *et al.* 2002), sleep and wakefulness (Takemiya, 2011), induction of fever (Scammell *et al.* 1996; Lazarus *et al.* 2007), synaptic plasticity and transmission (Akaneya & Tsumoto, 2006; Koch *et al.* 2010) and in the control of autonomic functions including respiration (Hofstetter *et al.* 2007).

Here we characterized the effects of PGE_2 on the preBötC using *in vitro* slice preparations and *in vivo* preparations from freely breathing animals. Our data demonstrate that low concentrations (<300 nM) of PGE_2 injected into the preBötC *in vivo* increased sigh frequency, but had no effect on normal breathing (eupnoea). Higher concentrations (1–2 μM) of PGE_2 were required to also increase eupnoeic frequency. This response was mimicked in the preBötC isolated in an *in vitro* brainstem slice preparation. Thus our study contributes to the notion that PGE_2 is an important modulator of respiratory activity. A comparison with existing studies (Ballanyi *et al.* 1997; Hofstetter *et al.* 2007) suggests that prostaglandins exert a variety of diverse effects that may result in adaptive and maladaptive responses of the respiratory network in health and disease.

Methods

All animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at Seattle Children's Research Institute. Mice were maintained with rodent diet and water available *ad libitum* in a vivarium with a 12 h light/dark cycle at 22°C.

In vivo recordings and microinjection into the preBötC

A total of six CD1 mice (postnatal days (P) 9–16) were anaesthetized with urethane (1.5 g kg^{-1}) by an intraperitoneal injection for *in vivo* measurements. At the end of the experiments animals were killed by transcardiac perfusion under terminal urethane anaesthesia. Mice, of either sex, were placed in a supine position, and the head was fixed with a stereotaxic apparatus. The neck of the mouse was opened from the ventral side, the trachea was cut, and plastic Y-shaped tubing for supplying oxygen was inserted into the proximal end of the trachea (cannulation). The bone of the skull covering the ventral brainstem was partially removed with small scissors and forceps. The dura and arachnoid membrane were removed to expose the ventral medulla. The surface of the ventral medulla was continuously perfused with 95% O_2–5% CO_2-equilibrated artificial cerebrospinal fluid (aCSF) solution at 30° ± 0.5°C. In all cases, 100% oxygen was supplied through cannulation without artificial ventilation. The core body temperature during *in vivo* experiments was measured routinely in our laboratory and was stable at 36° ± 1°C.

Electromyography recordings (EMG) of the intercostal muscles were recorded with a Teflon-covered Ag bipolar electrode. The skin over the abdominal and intercostal area on the right side was partially removed, and the bipolar electrode was placed on the surface of the intercostal muscles. Signals were AC amplified and bandpass filtered (8 Hz to 3 kHz).

Microsyringes (Hamilton microsyringe no. 80330, Hamilton, Reno, NV, USA) with 33 gauge needles containing PGE_2 were positioned with a micromanipulator for subsequent microinjections (KITE, World Precision Instruments, Sarasota, FL, USA). The needles of the microsyringes were inserted into the right preBötC from the ventral side. While constantly measuring intercostal EMG activity, PGE_2 was injected into the right preBötC (0.3 μl min^{-1}). We did not attempt any bilateral injections to limit the damage to the preBötC, which would have compromised respiratory rhythm generation. As previously described and anatomically characterized, injections in the preBötC are routinely traced and localized to an area encompassing the preBötzinger area in close proximity to the nucleus ambiguus (Zanella *et al.* 2014). As also demonstrated in previous studies, the injections typically encompass an area that slightly exceeds the preBötzinger area, and the reader is referred to the study by Zanella *et al.* (2014) for further details.

The transverse slice preparation

Brainstem transverse slice preparations from CD1 ($n = 65$) mice of either sex (P0–15, Charles River Laboratories International, Inc., Wilmington, MA, USA) were obtained as also described in detail previously (Ramirez *et al.* 1996). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Seattle Children's Research Institute. Mice were deeply anaesthetized with isoflurane (4%) before quick decapitation. Isolated brainstems were then placed in ice-cold aCSF bubbled with carbogen (95% O_2–5% CO_2). The aCSF contained (in mM): 118 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$, 0.5 NaH_2PO_4, and 30 D-glucose, pH 7.4. Brainstems were glued rostral end up onto an agar block for mounting into a vibratome (Leica Microsystems, GmbH, Wetzlar, Germany). A single 550–600 μm-thick slice was then taken. Slices were transferred into a recording chamber, continuously superfused with oxygenated aCSF, and maintained at a temperature of 30° ± 0.5°C. To initiate and maintain fictive respiratory rhythmic activity, the potassium concentration of the perfusate was raised from 3 to 8 mM over a period of 30 min. We routinely measured the P_{O_2} in the bath before, during and after the exposure to aCSF bubbled with 95% O_2 or 95% N_2 as described in detail in the study by Hill *et al.* (2011).

Extracellular population and intracellular current clamp recordings from the preBötC

In the transverse slice preparation, extracellular population recordings were obtained with suction electrodes positioned on the surface of the ventrolateral region containing the preBötC. To obtain a signal containing multi-unit action potential (AP) activity, extracellular signals were amplified 10,000-fold and filtered between 0.25 and 1.5 kHz using an AM instruments (A-M Systems, Sequim, WA, USA) extracellular amplifier (Fig. 1*B*, top trace). To facilitate the detection of bursts, this signal was rectified and integrated by using an electronic integrator with a time constant of 50 ms (Fig. 1*B*, middle trace) using home-built equipment. Intracellular current clamp recordings were obtained from respiratory neurons of the contralateral preBötC with the blind-patch technique. The patch electrodes were manufactured from filamented borosilicate glass tubes (Warner Instruments, Hamden, CT, USA 150TF), filled with a solution containing (in mM): 140 potassium gluconate, 1 $CaCl_2.6H_2O$, 10 EGTA, 2 $MgCl_2.6H_2O$, 4 Na_2ATP, and 10 Hepes (pH 7.2). In some cases the intracellular pipettes contained biocytin (4.5 mg ml^{-1}) to allow for the identification of neuron location and morphology. Recordings were low-pass filtered (0–2 kHz, Bessel four-pole filter, –3 dB). Neurons were identified as respiratory neurons by their discharge pattern in phase with the population activity of the contralateral preBötC (Fig. 1*B*, bottom trace).

Voltage clamp recordings of preBötC neurons

Whole cell patch clamp recordings of inspiratory neurons were obtained with a sample frequency of 10 kHz and a low-pass filter setting of 2 kHz. Recordings were made with unpolished patch electrodes, manufactured from borosilicate glass pipettes with a filament (Warner Instruments G150F-4, Warner Instruments, Hamden, CT, USA). The electrodes had a resistance of 3–5 MΩ when filled with the whole cell patch clamp pipette solution containing (in mM): 140 potassium gluconate, 1 $CaCl_2.6H_2O$, 10 EGTA, 2 $MgCl_2.6H_2O$, 4 Na_2ATP, and 10 Hepes (pH 7.2). The patch clamp experiments were performed with a patch clamp amplifier (Multipatch 700B, Molecular Devices, Sunnyvale, CA, USA), a digitizing interface (Digidata 1440A, Molecular Devices), and the software program pCLAMP 10.0 (Molecular Devices). Neurons located at least three to four cell layers (about 80–150 μm) caudal from the rostral surface of the slice were recorded under visual control. Neurons located directly at the slice surface were not examined because their dendritic processes were more likely to be damaged during the preparation than those of neurons located deeper within the slice. Current–response traces were recorded with either off- or online leak subtraction

(*P*/4 protocol), eliminating the linear leak current and residual capacity currents. The 2 mV liquid junction potential was manually subtracted with the amplifier's pipette offset regulator immediately before establishing the patch clamp configuration. The series resistance was always 80% compensated and regularly corrected throughout the experiments. We emphasize that whole cell voltage clamp recordings from neurons embedded in a functional network are accompanied by difficult space clamp control. This could lead to incorrect values for current amplitudes. Thus, recordings with obvious space clamp were discarded. Consulting equilibrium potential to holding potential relationships, all upward deflections had to be chloride-conducting, inhibitory postsynaptic currents (IPSCs), whereas all downward deflections had to be excitatory postsynaptic currents (EPSCs). EPSCs and IPSCs were analysed by using MiniAnalysis 5.41 (Synaptosft, Inc., Decatur, GA, USA) and statistical analysis was performed with Prism (GraphPad Software, Inc., La Jolla, CA, USA). The synaptic drive current in individual neurons, which occurred in phase with the rhythmic population discharges of the preBötC, was assessed by low-pass filtering the intracellularly recorded current traces (10 Hz) and determining the negative peak amplitude (maximum negative current during the burst). This amplitude was then compared during control conditions in the presence of 100 nM PGE_2 and in the presence of 2 μM PGE_2 (Fig. 6*Aa*).

Results

PGE_2 injections into the preBötC differentially affect sighs and eupnoeic breathing *in vivo*

In anaesthetized freely breathing CD1 mice (male and female, P9–16, $n = 6$) we tested the effect of microinjections of three different concentrations of PGE_2 (100 nM, 300 nM and 1 μM) into the right preBötzinger complex. The breathing was recorded with EMG recordings from the intercostal muscles (Fig. 1*A*, for details see Methods). At the lower concentrations we observed an increase in sigh frequency ($^*P < 0.05$, one-way ANOVA, Fig. 2*A* and *B*), while the eupnoeic frequency was unaltered (Fig. 2*B*). In contrast, higher concentrations of PGE_2 (1 μM) led to a significant increase in sighs and eupnoea compared to control conditions ($^*P < 0.05$, one-way ANOVA Fig. 2*B*).

PGE_2 application directly modulates the activity in the preBötC

Next we tested the effects of PGE_2 on the preBötC network in the isolated brainstem slice preparation of CD1 mice (male and female, P0–5). In a first set of extracellular experiments, the population activity of the preBötC was recorded with a surface electrode before and after bath application of PGE_2. Similar to the *in vivo* experiments we found a dose-dependent effect of PGE_2 on the fictive eupnoeic and sigh activities generated within the slices. At low concentrations (10–100 nM) PGE_2 strongly stimulated the frequency of sighs (Fig. 3*A* and *B*), without significantly affecting the amplitude, duration or frequency of eupnoeic bursts generated in the preBötC (Fig. 3*C*). At higher concentrations (2 μM) PGE_2 evoked a similar increase in sigh frequency as observed at lower concentrations, but PGE_2 had an additional enhancing effect on the eupnoeic frequency compared to control (Fig. 3*C*, $^{**}P < 0.01$, paired *t* test). The effects on both fictive eupnoea and fictive sighs were reversible upon washout.

PGE_2 increases the fictive gasping response of the preBötC to hypoxia

To assess if PGE_2 changes the activity of the preBötC during hypoxia, we quantified the response to severely reduced

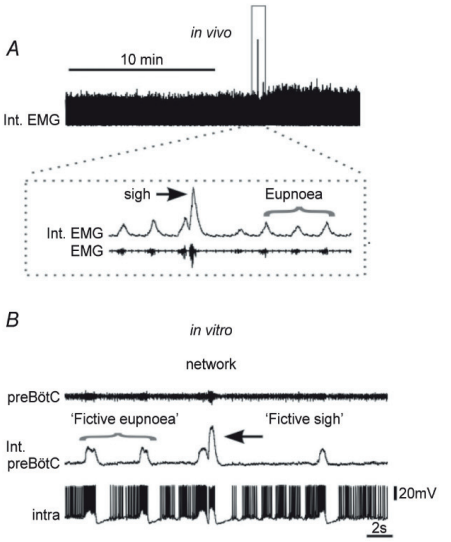

Figure 1. In vivo and in vitro measurements of distinct respiratory activities
A, a typical example of an EMG recording from a freely breathing anaesthetized mouse. In the top trace is shown the integrated and rectified trace (Int. EMG) of the raw EMG trace (EMG). The inset shows a typical example of a sigh interrupting eupnoea. B, multi-unit recording from a transverse brainstem slice preparation containing the preBötC (preBötC) and the integrated and rectified trace (Int. preBötC) and simultaneous intracellular recording (intra) of an inspiratory preBötC neuron in current clamp. Note that under control conditions the slices can generate two distinct activities ('fictive eupnoea' and 'fictive sighs').

levels of oxygen (95% N_2, 5% CO_2, Hill *et al.* 2011) in the absence and presence of different concentrations of PGE_2. For these experiments we applied PGE_2 for at least 10 min before the measurements. We characterized only one hypoxic exposure per slice. As previously described, exposure to hypoxia leads to a typical biphasic response with an early augmentation and a late depression phase in which the network reconfigures to generate fictive gasping (Fig. 4*A*, Telgkamp & Ramirez, 1999; Lieske *et al.* 2000; Peña *et al.* 2004; Hill *et al.* 2011). Slices exposed to moderate (100 nM, $n = 6$) or high (2 μM, $n = 6$) concentrations of PGE_2 (Fig. 4*B*, *C* and *D*) generated gasping activity with significantly increased amplitude and frequency values compared to untreated control slices (Fig. 4*D*, $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA). Slices exposed to the low concentration (10 nM) did not show a significantly different gasping compared to control slices ($n = 8$, one-way ANOVA).

Developmental changes in PGE_2 response

To determine if the effect of PGE_2 was dependent on the age of the animals we tested a set of slices obtained from animals during their second postnatal week (P7–12, $n = 6$ for 2 μM, $n = 4$ for 10–100 nM) and compared them to the data of the slices obtained during the first 5 days of life ($n = 6$ for 2 μM, $n = 14$ for 10–100 nM). The increase of the sigh frequency induced by a bath application of a low or high concentration of PGE_2 was significantly more pronounced in slices of mice prepared during the first postnatal week (Fig. 5*A*, two-way ANOVA, Holm-Sidak´s multiple comparison test). By contrast, slices obtained during the second postnatal week revealed only a slight increase at the low concentration (10–100 nM) and a slightly more pronounced effect in response to the high concentration of PGE_2 (2 μM) (Fig. 5*A*). High concentrations of PGE_2 significantly increased the frequency of fictive eupnoea in both age groups and no significant difference between the first compared to the second postnatal week was detected (Fig. 5*B*).

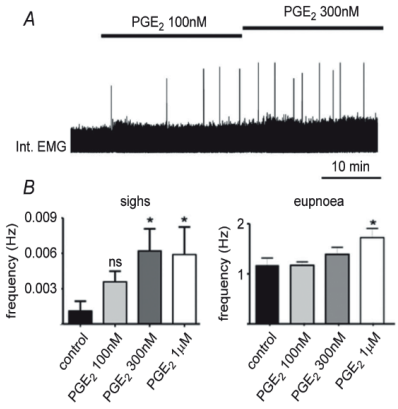

Figure 2. Microinjections of PGE $_2$ into the preB ötC increased the frequency of sighs and eupnoeic breathing at different concentrations
A, a typical example of the response to microinjections of PGE_2 (100 and 300 nM) into the preBötC of a freely breathing mouse. Note the increase of sighs at low concentrations (example trace in A and quantification in B). B, higher concentrations (1 µM) of PGE_2 injections increased the frequency of eupnoea and sighs ($^{*}P < 0.05$).

Spontaneous excitatory and inhibitory postsynaptic currents and the drive current

To test if PGE_2 affects synaptic transmission between respiratory neurons we recorded from inspiratory preBötC neurons in voltage clamp to measure the excitatory and inhibitory synaptic events (spontaneous (s)EPSCs and (s)IPSCs). Inspiratory neurons were defined as neurons that received phasic input during the population activity measured with a surface electrode on the preBötC (Fig. 6*Aa*). We measured sEPSCs and sIPSCs between the rhythmic population bursts before and after adding PGE_2 (100 nM and 2 μM) to the bath (Fig. 6*Ab* and *B*). As described in detail in the Methods section, we quantified amplitude, decay time and frequency of the EPSCs and IPSCs as an average of 50 events for each condition (control, 100 nM PGE_2, 2 μM PGE_2, $n = 5$ for all groups). PGE_2 led to a small, but significant reduction of the amplitude of sEPSCs ($^{*}P < 0.05$, one-way ANOVA) in the presence of 100 nM PGE_2, which returned to control values at the high concentration. All other measured parameters did not change when exposed to PGE_2 (Fig. 6*B*, one-way ANOVA). In addition, PGE_2 at high or low concentrations did not significantly change the drive current (Fig. 6*Aa*) compared to control conditions (Fig. 6*B*).

PGE_2 increases intrinsic bursting activity in inspiratory neurons

To test if PGE_2 modulates the intrinsic properties of preBötC neurons, we recorded from inspiratory neurons, which discharged in phase with the population burst recorded from the surface of the preBötC in the current clamp configuration (Fig. 7*A*). In a first set of experiments we tested the response of the neurons within the intact network ($n = 9$). Most neurons showed a slight depolarization of several millivolts in response to both tested concentrations (100 nM, $+4.75 \pm 3.10$ mV, $n = 4$; 2 μM, $+1.65 \pm 1.53$ mV, $n = 5$). However, there was no significant change in the drive potential underlying fictive eupnoea or fictive sighs (Fig. 7*A*). Next we tested

the effect on the neurons in isolation from synaptic inputs. All examined neurons ($n = 16$) showed either spontaneous tonic firing or intrinsic bursting firing (Fig. 7*B*, $n = 9$), when pharmacologically isolated from fast synaptic inputs by the addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM), CPP (10 μM), picrotoxin (5 μM) and strychnine (1 μM) to the bath. Similar to the neurons tested with intact synaptic transmission, PGE_2 caused a slight depolarization (2.65 $\pm$ 3.00 mV, $n = 16$). The depolarizations caused by either 100 nM or 2 μM PGE_2 were not significantly different in the tonic firing cells or intrinsic bursting neurons.

In intrinsic bursting cells we found a significant increase in the amplitude and frequency of the bursting after exposure to 2 μM PGE_2 (Fig. 7*C* and *D*, $^*P < 0.05$, paired *t* test, $n = 9$). It needs to be emphasized that bursting was irregular in some cases, and we also observed a substantial variability in the range of bursting in these cells. Thus, the evaluation of the burst frequency over a long period of time may not have been always consistent for all neurons. Previous studies identified two subtypes of pacemaker neurons based on the currents that are critical for the generation of the intrinsic bursts. Neurons that burst depending on the I_{CAN} were termed cadmium sensitive (CS), because bursting was blocked by cadmium, a blocker of calcium currents. Neurons with bursting properties that depend on the persistent sodium current were termed cadmium insensitive (CI) neurons, because their bursting persisted in the presence of cadmium (Thoby-Brisson & Ramirez, 2001; Peña *et al.* 2004). The stimulating effect of PGE_2 was found in CI pacemaker neurons ($n = 4$). These neurons were sensitive to 10 μM riluzole or insensitive to 200 μM Cd^{2+}. A stimulating effect of PGE_2 was also found in CS neurons ($n = 3$). These neurons were sensitive to 200 μM Cd^{2+}. In two neurons the response to Cd^{2+} or riluzole was not further tested. Since we only tested the high concentration of PGE_2 we were not able to distinguish from these experiments whether a specific cell type was essential for the specific effect of PGE_2 on sigh frequency. We therefore conducted additional extracellular experiments to assess the effect of these blockers at the network level.

Differential contribution of I_{CAN} and I_{Nap} to the PGE_2 effect

As mentioned above, it was previously shown that intrinsic bursting properties in the inspiratory neurons of the preBötC can depend either on the persistent sodium current (I_{Nap}) or on a calcium-activated non-selective cation conductance (I_{CAN}) (Peña *et al.* 2004). To test if I_{CAN}- or I_{Nap}-dependent bursting plays a critical role in

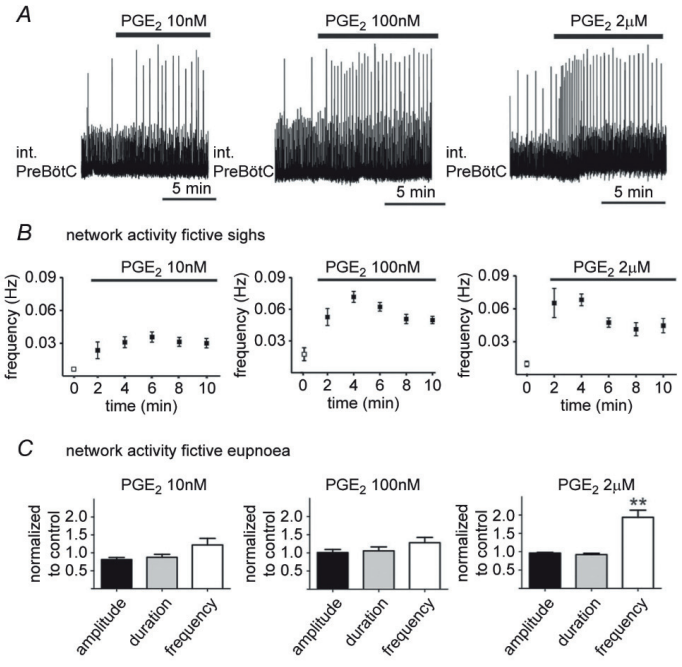

Figure 3. Bath applications of PGE_2 increased the frequency of fictive sighs and eupnoea at different concentrations
A, typical extracellular recordings from the preBötC of an isolated brainstem slice preparation in response to bath application of PGE_2 (10 nM, 100 nM and 2 µM). B, fictive sigh frequency increased in response to PGE_2 at both low (10 nM (n = 8), 100 nM (n = 6)) and high concentrations (2 µM, n = 6). C, only high concentrations (2 µM) PGE_2 exposure led to a significant increase in eupnoeic burst frequency compared to control ($^{**}P < 0.01$, paired t test), while at all concentrations no change in amplitude or duration of the eupnoeic bursts was observed.